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Lipid Composition and Acid Hydrolase Content of Lamellar Granules of Fetal Rat Epidermis*

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Lipids and acid hydrolases have been characterized in a subcellular fraction, enriched with lamellar granules (LG), derived from fetal rat epidermis. This fraction contains 23% glycosyl ceramides and ceramides, 15% free sterols, and 34% phospholipids. The lipid/protein ratio is 2.0. The sterols and sphingolipids were present in proportions similar to those previously reported in stratum corneum. These findings provide direct biochemical evidence for the widely accepted hypothesis that stratum corneum lipids are derived from exocytosis of lamellar granules into the intercellular space. The LG fraction was enriched in certain acid hydrolases including glucosidase, acid phosphatase, phospholipases A, and sphingomyelinase; other acid hydrolases, i.e., aminoglycosidases, glactosidase and aryl sulfatase (pH 5.5), and steroid sulfatase were not preferentially localized in this fraction. By modulation of phospholipids, glycolipids, and proteins in the membrane regions of stratum corneum, the acid hydrolases of LG may play a role relevant to the function and desquamation of stratum corneum.

It has been inferred from considerable biochemical and cytochemical evidence that the lipids of stratum corneum largely reside in the intercellular and membrane regions of this layer [1,2]. Their presence is attributed in large measure to the extruded content of lamellar granules (LG) and is presumed to reflect the fusion of the individual lipid-rich lamellae into broad sheets [1–3]. The lipids of stratum corneum are characteristic of that tissue and differ significantly from the lipids of basal cells and spinous layer cells in their high content of sphingolipids and sterols and their low to absent content of phospholipids [3,4]. This general pattern has been demonstrated for rodent, pig, and human epidermis [5].

Although it is postulated that the intercellular lipids of stratum corneum originate in LG, there have been no direct chemical examinations of LG lipids. Histochemical studies have suggested they contain sterols, phospholipids, and glycolipids [6–8]. More direct examination has been impossible because LG have not been isolated. Furthermore, LG have been classified as lysosomes on the basis of histochemically demonstrated acid phosphatase (and possibly other acid hydrolases) [9]. Their extrusion into the intracellular space appears to be linked to the presence of acid phosphatase in this domain. Recent studies have suggested that other hydrolytic processes are ongoing in the intercellular space of stratum corneum, raising the possibility that hydrolytic enzymes may originate in LG which can modify the content of the intercellular space and contribute to desquamation and permeability characteristics of the stratum corneum [9–11].

We have previously reported on a subcellular fraction enriched in LG, bearing acid phosphatase, isolated from fetal rat epidermis [12]. We have subsequently shown that this fraction is enriched in glycosphingolipids and ceramides [13]. These lipids are believed to play a major role in forming the broad intercellular lipid-containing sheets in stratum corneum and their contribution to the water barrier has been discussed [3,14,15]. The LG fraction was also enriched in acylglucosylceramide, a major component of epidermal glycosphingolipids that has been speculatively implicated in the formation of the stacked discs of LG [16,17]. A similar fraction has been more recently isolated from newborn mouse skin and also is enriched in acid phosphatase and sphingolipids [18]. In the present report we present the first extensive quantification of the acid hydrolases and the general lipid composition of LG.

MATERIALS AND METHODS

Tissue Fractions

Skin was obtained from fetal Sprague-Dawley rats at 20 days of gestation. Epidermis was separated after incubation of whole skin in 0.1 M dithiothreitol in minimum essential medium for 30 min at 37°C. Homogenates of 1.0–1.5 g epidermis in 0.25 M sucrose (100 mg/ml) were fractionated as previously described [12]. Briefly, a pellet was obtained after centrifugation of a 700 *g* supernatant at 17,000 *g*. We have previously shown that LG are sedimented at 17,000 *g* (17K) and cannot be demonstrated in significant quantities in sediments obtained from the 17K supernatant at greater centrifugal force [12]. The 17K pellet thus served as the starting fraction for subsequent purification of LG. The pellet was resuspended in 0.5 ml 0.25 M sucrose and fractionated on a continuous gradient of metrizamide (analytical grade Nyegaard and Co, Oslo), 20–50%. The 17K fraction and particulate fractions from the gradient were washed with 0.25 M sucrose and repelleted at 19,000 *g* for subsequent assays of enzyme activity and analysis of protein and lipid content. Morphology of the fractions was monitored by electron microscopy as previously described [12].

Lipid Content

Total lipids were extracted by the Folch method [19] and further fractionated by thin-layer chromatography (TLC) on silica gel 60 (E. Merck, Darmstadt) using two 2-dimensional systems as follows:

System I: Lipids were quantitatively applied to prescored plates and developed in ethylacetate:benzene (1:1) in the first dimension followed by chloroform:methanol:acetic acid:water (104:40:14:6) in the second dimension. This system resolved lysophosphoglycerides, sphingomyelin, phosphoglycerides, glycosphingolipids, ceramides, and free sterols. Other neutral lipids were only partially resolved and not further identified.

System II: Lipids were fractionated on prescored plates using chloroform:methanol:acetic acid:water (104:40:14:6) in the first dimension. After rinsing with acetone, plates were developed in the second dimension with chloroform:methanol:water:40% methylamine (65:35:5:5). This system provided better discrimination of the individual phospholipids found in System I.

Lipid spots were identified by cochromatography of authentic standards. Phospholipids, sphingomyelin, sterols, and ceramides were obtained from Sigma. Glucosylceramide and acylglucosylceramide derived from pig epidermis were kindly supplied by Dr. P. W. Wertz. Lipids

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Abbreviations:

LG: lamellar granule(s)

PC: 1,2-di([1-^{14}C]oleoyl)phosphatidylcholine

TLC: thin-layer chromatography

were also identified by tinctorial properties: chlorox-benzidine for sphingolipids, Bial's orcinol reagent for glycolipids, Dragendorff's reagent for choline, molybdenum blue for phosphorus-containing lipids and ninhydrin for nitrogen-containing lipids (reagents from Applied Science Co.)

For quantitation, lipid spots, identified with iodine vapors, were scraped from the plates. Total and individual lipids were quantitated by a modification of the method of Amenta [20] in which lipids are eluted from silica gel with chloroform methanol (1:1) at 45°C and then reacted with dichromate-H_2SO_4. Standard curves with cholesterol, ceramides, cerebrosides, and phospholipids were run simultaneously since each class gives slightly different results. Total lipid concentration was estimated against a standard arbitrarily composed of 25% phospholipid, 25% cholesterol, 10% cerebroside, 15% ceramide, and 25% triglyceride. The method was sensitive over two convenient ranges, 10–60 μg and 100–300 μg, and reproducible with an error of less than 10%. Proteins were determined by the method of Lowry et al [21] on samples digested in NaOH.

Enzymes

Enzymes were assayed in aliquots of subcellular fractions containing 20–200 μg protein. For each enzyme, specific activity per mg protein was calculated from the units of activity.

Acid hydrolases in subcellular fractions were assayed using paranitrophenyl substrates (Sigma) in concentrations and at the pH shown in Table I. Assays were conducted as previously described in a system containing 0.05% Triton-X 100, 0.05 M acetate buffer in a total volume of 0.5 ml for 30 min at 37°C [22,23]. Reaction products were determined spectrophotometrically and expressed as arbitrary units of optical density. Appropriate substrate concentrations and linearity of the reactions were determined in preliminary experiments.

Phospholipase A was assayed as previously described [24] using 1,2-di([1-^{14}C]oleoyl)phosphatidyl choline (PC) (Amersham Searle) as substrate. Assays were conducted with 0.5 μCi PC (0.34 mM) in a system containing 0.25% taurodeoxycholate, 0.05 M acetate buffer, pH 4.6, in a volume of 0.5 ml for 45 min at 37°C. Activity of phospholipase A was calculated as % substrate hydrolyzed to lysophosphatidyl choline and fatty acid. Sphingomyelinase was assayed by the method of Bowser and Gray [25] using [N-methyl-^{14}C]sphingomyelin (Amersham-Searle) 1 μCi, 0.34 mM in 0.25% Triton-X 100, 0.05 M acetate buffer, pH 4.8, in a volume of 0.5 ml for 1 h at 37°C. Results were expressed as % water soluble radioactivity (largely [^{14}C]phosphorylcholine) liberated from the substrate. Steroid sulfatase was assayed by the method of Ruokonen and Oikarinen [26] using [7-^{3}H]dehydroepiandrosterone sulfate (New England Nuclear). 500,000 dpm (sp act 25 μCi/mmol) were incubated with subcellular fractions in phosphate-buffered saline at pH 7.3 in a total volume of 0.7 ml for 2 h at 37°C. Free ^{3}H-labeled steroid was quantitatively extracted in ethylacetate:ethyl ether (1:9) and results expressed as % of substrate hydrolyzed.

RESULTS

The lipid composition and enzyme activity were characterized in the 17K starting fraction and the 3 subcellular fractions obtained from 17K. The 17K fraction contained LG, mitochondria, cornified cell envelopes, vesicular structures, tonofilaments, and amorphous debris. The subsequent fractions included a very-low-density fraction enriched in LG, a fraction containing small well-preserved mitochondria contaminated by very few vesicles and a small amount of amorphous debris and denser fraction containing mitochondria, cornified cell envelopes, and debris (fraction III).

TABLE I. *Assay of hydrolases*

Enzyme	Substrate	Conc. (mM)	pH
Acid phosphatase	pNP phosphate	6	4.5
Aryl sulfatase	pNP sulfate K	5	5.5
α Glucosidase	pNP D glucoside	1	5.0
α Galactosidase	pNP D galactoside	2	5.0
Glucosaminidase	pNP N-acetylglucosamine	4	4.5
Galactosaminidase	pNP N-acetylgalactosamine	4	4.5
Phospholipase A	1,2-di([1-^{14}C]oleoyl)-phosphatidylcholine	0.34	4.6
Sphingomyelinase	[N-methyl-^{14}C]sphingomyelin	0.34	4.8
Steroid sulfatase	[7-^{3}H]dehydroepiandrosterone sulfate	0.014	7.3

Lipids

Lipids of each fraction were isolated by TLC and identified as specified in the *Materials and Methods*. The lipids of the LG fraction are represented in Fig 1. Phospholipids, which were further resolved in TLC system II, included phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin, and lysophosphatidylcholine plus several minor phospholipids that were not identified (e.g., spot #5 in Fig 1).

Glycolipids in LG were present as 3 spots in the TLC system used; 2 had the mobility of glucosylceramides and the least polar (spot #8) cochromatographed with acylglucosylceramide. Ceramides were present as 5 separate spots (9–13). We have previously reported resolution of 4 glucosylceramides and 6 ceramides from LG lipids [13] using several different systems of TLC which provide greater discrimination of each type of sphingolipid. For purposes of this study we used 2-dimensional TLC to provide quantitation on a single plate. Free sterols were present in spot #14.

The content of lipid and protein of each fraction was quantitated (Table II). LG had a higher lipid/protein ratio than the other 2 fractions and the crude starting 17,000 *g* (17K) fraction. The lipid/protein ratio of 2.0 was consistent with its low buoyant density in metrizamide (0.8–1.0) [12]. Approximately 45% of the lipid in the 17K pellet was recovered in the 3 particulate fractions and of this 14% was present in LG.

Individual lipid components were quantitated in the various subcellular fractions (Table III). The LG fraction most resembled the 17K strating fraction and was rich in sphingolipids including sphingomyelin, glucosylceramides, and ceramides which accounted for almost 33% of the total lipids in this

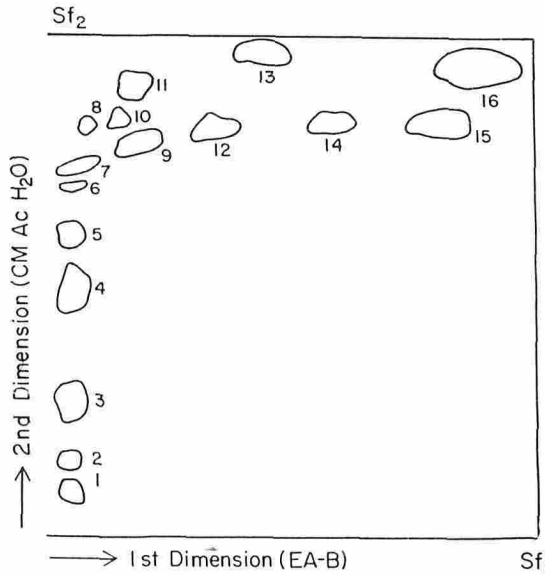

FIG 1. *Tracing of a two-dimensional thin-layer chromatogram* using system I as outlined in the text. Plate developed in the first dimension in ethylacetate benzene (*EA-B*) 1:1 to solvent front (Sf_1) and then in the second dimension in chloroform:methanol:acetic acid:water (*CM Ac* H_2O) 104:40:14:6 to Sf_2. Lipids identified, as described in text, as follows: *spot #1*, lysophosphatidylcholine; *#2* sphingomyelin; *#3 and 4*, phosphoglycerides including phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol; *#5*, unidentified phospholipid; *#6 and 7*, glucosylceramides; *#8*, acylglucosylceramide; *#9–13*, ceramides; *#14*, sterol; *#15 and 16*, neutral lipids not definitively identified.

TABLE II. *Lipid content of subcellular fractions*

Fraction (n)[a]	mg/g Epidermis ±SD[b]	mg/mg Protein ±SD[b]	Percent of total lipid
17K (6)	1.811 ± .188	0.388 ± .020	100
LG (6)	0.260 ± .132	2.000 ± .664	14.3
Mitochondria (2)	0.070	0.890	3.8
Fr III (2)	0.490	0.460	27.0

[a] n = Number of experiments, each utilizing 1200 mg of fetal rat epidermis for separation of fractions into a 17,000 *g* particulate fraction (17K), subsequently subfractionated into lamellar granule (LG), mitochondrial, and mixed mitochondrial, cell envelope (Fr III) fractions.

[b] Lipids and proteins were assessed in washed particulate fractions obtained as per text.

TABLE III. *Lipid composition of subcellular fractions*

Lipid	Percent of total lipid ± SD			
	17K[a]	LG	Mitochondria	Fr III
Phosphoglycerides[b]	26.5 ± 1.5	24.5 ± 1.3	60.8 ± 5.1	10.4 ± 1.8
Sphingomyelin	5.3 ± 0.2	9.4 ± 0.3	—	2.6 ± 1.0
Glycosphingolipids and ceramides	28.8 ± 1.4	23.4 ± 1.0	—	16.3 ± 2.3
Sterols	14.5 ± 2.4	15.2 ± 2.6	30.8 ± 8.2	11.7 ± 3.8
Total	75.0	72.5	91.5	41.0

[a] Results represent the mean of 4 experiments as % of total lipid ± SD. Fractions 17K, LG, mitochondria, Fr III are as defined in Table II.

[b] Phosphoglycerides are all phosphorus-containing lipids except sphingomyelin.

TABLE IV. *Acid hydrolases of subcellular fractions*

	Relative specific activity ± SD[a]		
	LG	Mitochondria	Fr III
Acid phosphatase	7.1 ± 1.9[b]	0.1	1.0 ± 0.7
Arylsulfatase	0.8 ± 0	0	1.0 ± 0.8
Galactosidase	1.4 ± 0.2	0	1.1 ± 0.1
Galactosaminidase	0.8 ± 0.1	0	0.9 ± 0.8
Glucosidase	2.0 ± 0.4[b]	0	0.8 ± 0.8
Glucosaminidase	0.8 ± 0.4	ND	0.9 ± 0.3
Acid phospholipase A	3.6 ± 1.3[b]	2.6 ± 0.2[b]	1.6 ± .05[b]
Sphingomyelinase	4.0 ± 0.8[b]	0.6 ± 0	0.3 ± 0.2

[a] Results represent the mean ± SD of 3 experiments and are expressed as the mean of the ratios of the specific activity of the enzyme in each fraction/specific activity in 17K in each experiment. Experiments were performed as per text using washed, repelleted subcellular fractions for enzyme assays and protein analysis. Abbreviations as in Table II.

[b] Denotes statistically significant increases in relative specific activity ($p < .05$ by Student's *t*-test).

fraction. Mitochondria differed markedly, consisting mainly of sterols (30%) and phospholipids (60%). Fraction III, the mixed fraction containing cornified cell fragments, had smaller amounts of phospholipids; moreover the amount of glucosylceramides and ceramides was greater relative to phospholipids than in the LG fraction.

Enzymes

All of the enzymes we assayed were present both in the 17K pellet and the supernatant fraction. To assess specific localization of an enzyme, we compared its specific activity in the washed particulate fraction to that in 17K (Table IV). In this convention, a ratio of activity in the fraction vs that of 17K greater than 1.0 would suggest localization of the enzyme to that fraction. Of the 8 acid hydrolases, 4 showed greater specific activity in the LG fraction than in 17K, including acid phosphatase, glucosidase, acid phospholipase A, and sphingomyelinase. Of these 8 enzymes, only the phospholipase could be demonstrated in significant quantities in mitochondria, and acid phospholipase showed some localization as well in fraction III. Otherwise, no enzymes appeared to be specifically localized to this mixed fraction.

TABLE V. *Steroid sulfatase activity*

Subcellular fractions[a]	Percent activity[b]	Relative specific activity[c]
17,000 *g* Supernatant	71.6	ND
17,000 *g* Pellet	28.4	1.0
LG	0.3	0.34
Mitochondria	0.5	0.23
Fraction III	5.3	10.7

[a] Fractions prepared as per text; abbreviations per Table II. Approximately 1.0 g epidermis was used in this representative experiment.

[b] Percent total activity found in each fraction derived from 700 *g* supernatant. Assays conducted as per text with 45–150 μg protein in each of duplicate reaction vessels. Activity expressed as % of starting dpm in [^{3}H]dehydroepiandrosterone converted to free steroid.

[c] Relative specific activity calculated as in Table III; i.e., ratio of activity/mg protein in each fraction relative to that in 17,000 *g* pellet.

We also examined the fractions for presence of steroid sulfatase (Table V). Like all other enzymes it was present in both 17K pellet and supernatant. It was not specifically localized in either LG or mitochondria but showed a 10-fold increase in specific activity in fraction III as compared to 17K.

DISCUSSION

We have recently demonstrated the presence of the glucosylceramides and of acylglucosylceramide typical of stratum corneum in the rat epidermal LG [13]. In this report we confirm the abundance of lipid and presence of a variety of acid hydrolases in this fraction. Our data show that LG seem to be composed primarily of lipids with a low content of protein as would be expected if they are assembled from stacks of flattened liposomes as has been suggested [16,17].

Taken together, the above findings provide the first direct biochemical evidence in support of the hypothesis that stratum corneum lipids derive from the exocytosis of LG. Although phospholipids are markedly decreased in rodent stratum corneum, their presence in LG is hardly surprising. LG are bounded by limiting membranes derived from the Golgi apparatus and thus are likely to be rich in phospholipids. These limiting membranes are probably retained in the granular cell when the granules fuse with plasma membranes for exocytosis of their lamellated contents. Moreover, other phospholipids contributing to the stacked discs may be hydrolyzed after extrusion (see below). If one considers the composition of the LG lipids without phosphoglycerides, free sterols account for 21%, glycolipids and ceramides for 31%, and sphingomyelin for 12%; these proportions are close to those reported for rodent stratum corneum [3].

It should be noted that although LG lipids are very similar in distribution to those of the crude 17K fraction, we recovered only 14% of 17K lipids in the LG fraction. We believe that this is in part due to presence of stratum corneum in 17K but as well to the poor yield of LG after even mild processing. Our previous studies of the 17K fraction suggested it was greatly enriched in LG but was contaminated with mitochondria and some remnants of cornified cells [23]. The procedure used for isolating LG removes these contaminants but apparently also causes significant losses of LG.

These studies have also demonstrated concentrations of certain specific lysosomal type hydrolases in the LG fraction. In addition to acid phosphatase [12] we have now shown localization of glucosidase, acid phospholipase A, and sphingomyelinase in the LG fraction. On the other hand, we failed to demonstrate preferential localization of other acid hydrolases such as arylsulfatase and other glycosidases. We have not as

yet examined the presence of proteolytic enzymes or glucuronidase. The latter, at least, is not localized in the starting fraction: 17K pellet [23].

Arylsulfatase (pH 5.5) has been demonstrated in LG by ultrastructural histochemical techniques in mouse epidermis [27]. In other studies, it could not be shown to be present [28]. Our failure to localize it in the G fraction must therefore be viewed somewhat tentatively since it may be present in too few of the LG [27] to allow for biochemical localization in the presence of the enzyme in other organelles.

However, it does not seem fortuitous that the enzymes we found were glucosidase and acid phospholipases. It is likely that these enzymes serve specific functions with respect to the content of LG. Ceramides are more abundant in relation to glycolipids in stratum corneum than in granular cells of murine epidermis [3]. Similarly, the relationship between the glucosylceramides and ceramides of LG vs stratum corneum of rat epidermis has suggested that hydrolysis of the former gives rise to the latter [13]. Such remodeling may be facilitated by the glucosidase and phospholipases that are probably extruded into the extracellular space together with the lipid-containing discs. How this correlates with barrier function and desquamation remains to be determined. In this regard, a recent report that stratum corneum cells fail to reaggregate after treatment with α and β glucosidases may be of interest [11].

No function has yet been ascribed to the acid phosphatase present in LG and readily demonstrable in the extracellular spaces of stratum corneum layers. Since acid phosphatase is a characteristic enzyme of many lysosomal organelles, it is possible that in the case of LG it represents a nonfunctional marker of the lysomal origin of the granule.

Finally, we were unable to demonstrate localization of steroid sulfatase activity among the hydrolases of LG although it was abundantly present in 17K supernatant and in fraction III which is enriched with stratum corneum membranes. These findings are consistent with published evidence that the enzyme is localized in microsomal membranes [29] and stratum corneum [30]. Although apparent lack of activity does not exclude the possibility that the enzyme is packaged in LG in an inactive form and activated only after extrusion, the present findings do not shed any light on the mechanism by which steroid sulfatase might find its way to the membrane regions of stratum corneum as has been reported [30].

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